TITLE: BETA-EXPANSINS AS CELL WALL LOOSENING AGENTS, COMPOSITIONS THEREOF AND METHODS OF USE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 U.S.C. § 119 of a provisional application Ser. No. 60/045,445 filed May 2, 1997, and from U.S. Application 09/071,252 filed May 1, 1998, which applications are hereby incorporated by reference in their entirety.

GRANT REFERENCE

This research was supported by the grants MCB-9317864 from the US National Science Foundation. The United States Government may have some rights in this invention.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to proteins belonging to a novel class of proteins designated as β-expansins, a composition comprising such proteins, isolated polynucleotides encoding β-expansins, methods for using the polynucleotides and proteins of the invention and methods for identifying, isolating and purifying expansins, including α and β-expansins.

Background of the Invention

Many grasses, such as rye grass, Kentucky bluegrass and orchard grass, release prodigious quantities of wind-dispersed pollen that trigger hayfever. Seasonal asthma and related immune reactions in humans. Up to 25% of adults suffer these allergic responses as a result of inhaling pollen-laden air. (Knox, B. et al., (1996) Trends in Plant Science 1:156-164.) The major and most wide-spread allergenic component of grass pollen are the group I allergens. (Griffith, I., et al, (1991) FEBS Lett. 279:210-215; Perez, M., et al., (1990) J. Biol. Chem. 265:16210-16215; Esch, R. E. et al., (1989) Mol. Immunol. 26:557-561.) These are glycoproteins of about 30 kD that are quickly and profusely released by grass pollen upon hydration; in humans they bind to IgE antibodies to initiate the allergic response. Pollen from grasses contain one or more forms of these allergens, which are named after the source species, e.g. Lol pI is from Lolium perenne (rye grass), Ory sI is from Oryza sativa (rice), etc. Although the immunological aspects of these allergens, especially Lol pI, have been extensively studied, their biological function in the

plant is unknown. Nevertheless, high sequence conservation among homologs in divergent grass species implies that they serve a vital biological function. (Xu, H. L., et al., (1995) Gene 164:255-259; Broadwater, A. H., et al., (1993) Gene 131:227-230.)

Recently, Shcherban et al. (Shcherban, T. Y., et al., (1995) Proc. Natl. Acad. Sci. USA, 92:9245-9249) noted that group I pollen allergens have a distant sequence similarity to expansins. Expansins are extracellular proteins that promote plant cell wall enlargement, evidently by disrupting noncovalent bonding between cellulose microfibrils and matrix polymers. (McQueen-Mason, S., et al. (1994) Proc. Natl Acad. Sci. USA 91:6574-6578; McQueen-Mason, S. et al., (1992) Plant Cell 4:1425-1433.) These previously described expansins are referred to in this specification as alpha-expansins. Applicant has now surprisingly discovered that the group I pollen allergens are structurally and functionally related to expansins and that they comprise a second family of expansins, β-expansins.

BRIEF SUMMARY OF THE INVENTION

The present invention relates to β-expansins, including vegetative homologs of β-expansins, compositions thereof and isolated polynucleotides encoding the β-expansins of the invention. Beta-expansins, and polynucleotides encoding β-expansins, of the invention may be of natural origin, isolated and purified or recombinately produced. For purposes of the present invention, a "vegetative homolog" is defined as a β-expansin which is originally found in any plant part but pollen.

In one aspect, the invention relates to a polypeptide belonging to a class of β-expansins such as, for example, a group I grass pollen allergen and a vegetative β-expansin and compositions thereof.

In another aspect, the invention relates to a polynucleotide encoding the β-expansin of the invention, and a vector, a host cell and a transgenic plant comprising said polynucleotide.

In yet another aspect, the invention relates to a method of altering physical properties of the plant cell wall or any cell wall products derived from plant material, for example paper or textile.

In a further aspect, the invention relates to a method of identifying, isolating and purifying an expansin protein (including both α and β-expansins) or a polynucleotide encoding such protein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a structural comparisons of alpha-expansins and group I allergens. (A) Dot plot of Cs-EXP 1 (Genbank U30382) with Lol pI (Genbank X57678) shows limited, but distributed, sequence similarity. Alignments of the most conserved regions (I to 5) are shown at right. Conserved amino acids are underlined in the top line and represented by a period (.) in the lower line. (B) Secondary structure predictions for expansins and allergens show close similarity between these two groups of proteins. Regions of the protein with a PHDsec score < 7 are shown as open (unpatterned) blocks. Arrows mark two notable disagreements in the predicted structures. The expansin prediction was based on Genbank sequences U30460, U30476, U30477, U30478, U30479, U30480, U30381, U30382, X85187, Y07782, U85246. The allergen prediction was based on Genbank sequences U31771, M57474, U03860, L14271, X78813, Z27084, A31060, Z27090. Signal peptides were removed from the sequences before analysis. (C) Hydrophobic cluster analysis of the allergen Lol pl and expansin Cs-EXP I indicates good concordance between the two proteins. Shaded boxes demarcate putative homologous hydrophobic clusters. Domains of high sequence similarity are outlined with dotted lines and were used as "landmarks" to identify homologous clusters. The conserved regions in A are also indicated in B and C (numbered 1 to 5). The conserved cysteines are found in the dotted regions 1, 2, and 4.

Figure 2 shows enhancement of cell wall extension (top panels) and stress relaxation (bottom panels) by maize pollen extract. (A) and (B) show rheology responses of maize silk walls to pollen extract diluted to 20% strength (1:4 dilution with 50 mM acetate buffer, pH 4.5). (C) and (D) show responses of wheat coleoptile walls to 20% pollen extract. (E) and (F) show the modest responses of cucumber hypocotyl walls to undiluted (100%) pollen extract. For the extension assays, heat-inactivated wall specimens were clamped in a constant-load extensometer in 50 mM sodium acetate buffer, pH 4.5; wall extension (creep) was detected by a position transducer attached to one of the clamps and is plotted as extension rate. (McQueen-Mason, S. et al., (1992) Plant Cell 4:1425--1433; Cosgrove, D. J. (1989) Planta 177:121-130.) At the time indicated by the arrow, the buffer surrounding the wall specimen was exchanged for a similar one containing maize pollen extract. Extension traces show two representative results from 4 to 8 replicates. For the stress relaxation assays, heat-inactivated walls were pre-incubated in buffer +/- pollen extract, then clamped in an

extensometer, extended to a predetermined load, and held at constant length during the subsequent relaxation (Cosgrove 1989) either in 50 mM acetate buffer (dotted lines) or the same buffer containing maize pollen extract at the dilution indicated. The decay in stress is plotted as a relaxation spectrum (log-time derivative of stress). Each relaxation curve is the average of 6-9 independent relaxation measurements.

Figure 3 shows dentification of Zea mI in maize pollen extracts and its association with wall extension activity. (A) Coomassie-stained SDS polyacrylamide gel of total proteins eluted from maize pollen. (B) Western blot of total proteins eluted from maize pollen, using rabbit polyclonal antibodies against cucumber ("Sl", Cs-EXPl expansin (Shcherban 1995; Li, Z.-C., et al. (1993) *Planta* 191:349-356, 14). (C) Western blot of total proteins eluted from maize pollen, using monoclonal antibody directed against a Lol pI fragment (site D). (Esch 1989). (D) Fractionation of maize pollen protein on CM-Sepharose. (E) Coomassie-stained SDS-PAGE and (F) western blot of CM-Sephadex fraction (f7) with highly purified Zea mI protein. (G) Extension curve of heat-inactivated walls of maize silks treated (arrow) with purified Zea mI (fraction f7) brought to pH 4.5 with sodium acetate buffer.

Figure 4 shows phylogenetic tree of α-expansins, group I allergens, and their vegetative homologs. Protein sequences were aligned using the Clustal program with PAM250 weight table and the tree was constructed by bootstrap analysis (1,000 replications) using nearest neighboring joining of the Poisson-Corrected values for amino-acid differences, using the MEGA phylogenetic analysis program (S. Kumar, K. Tamura and M. Nei, Institute for Molecular Evolutionary Genetics, Pennsylvania State University). The numbers on the tree indicate the bootstrap P-values. Genbank accession numbers are also indicated for each sequence.

Figure 5 shows an amino acid alignment of four β and four α-expansins prepared using the Clustal algorithm. Strictly conserved residues are boxed in. The following sequences are represented: Lolpl.PRO (Genbank M57494); OsEXPlbeta.PRO (Genbank U95968); CuEXPISIGN.PRO (Genbank U30482); CuEXP2sign.PRO (Genbank U30460); OsEXP1.PRO (Genbank Y07782); Z37641.PRO (Genbank U95967).

Figure 6 shows an amino acid alignment of seven β-expansins prepared using the Clustal algorithm. Strictly conserved residues are boxed in. The following sequences are represented: z37641.PRO (Genbank U95967); zeamla.PRO (Genbank L14271); Lolpl.PRO (Genbank M57474); OsEXPlbeta.PRO (Genbank U95968); ciml.PRO (Genbank U03860);

beta2 predicted protein (unpublished) SEQ ID NO:16; and Z37641.PRO (Genbank U95967).

DETAILED DESCRIPTION OF THE INVENTION

All patents, patent applications and publications cited herein are hereby incorporated by reference. In case of inconsistencies the present disclosure governs.

The present invention relates to proteins belonging to a novel class of proteins designated as β-expansins, a composition comprising such proteins, polynucleotides encoding β-expansins (and vectors, host cells and plants containing such polypeptides), a method for using the polynucleotides and proteins of the invention, and a method for identifying, isolating and purifying expansins, including both α and β-expansins. Beta-expansins of the invention, and polynucleotides encoding β-expansins, may be of natural origin, isolated and purified or recombinatly produced.

A polypeptide of the invention, referred to as a β-expansin, is a polypeptide having a molecular weight from about 24 kD to about 35 kD, preferably from about 24 kD to about 32 kD, and most preferably from about 25 kD to about 28 kD. When the molecular weight of the protein of the invention is measured by SDS-PAGE using the conditions set forth in the Example, the molecular weight of the polypeptide may be from about 25 to about 35kD. The polypeptides of the invention include but are not limited to class I pollen allergens and vegetative homologs of such allergens. Beta-expansins of the invention have the property of altering physical properties of a plant cell wall. For purposes of the present disclosure, "altering physical characteristics of a plant cell wall" includes loosening or expanding cell walls, altering cell wall mechanical strength, altering the bonding relationship between the components of the cell wall and/or altering the growth of the plant cell wall. This property of β-expansins of the invention may be determined by using assays well known in the art, such as cell-wall extension and stress relaxation assays. Induction of cell wall extension (creep) and an increase in the stress relaxation spectrum of the wall are diagnostic for expansins (both α and β). Expansins show an effect in these assays at, for example, a dosage of 1 part (and above) protein to 1,000 to 10,000 parts cell wall (on a dry weight basis).

Beta-expansins of the invention are similar to α-expansins described in co-pending U.S. applications Ser. Nos. 08/834,327 filed April 15, 1997 and 08/440,517 filed May 12, 1995 in that they both have the property of inducing stress relaxation and extension of plant cell walls. However, β-expansins have low amino acid sequence similarity with α-expansins,

which is about 25% as determined by BLAST or FASTA algorithms. Furthermore, β-expansins are more effective on grass cell walls than on dicotyledon plant cell walls. In contrast, α-expansins are more effective on dicotyledon plant cells walls than on grass cell walls. Since it is known that monocot and dicot cell walls differ in their chemical composition, it is likely that β and α-expansins act on different components of the plant cell wall.

Beta-expansins of the invention are characterized by the following conserved structural elements. With respect to the primary structure, the amino acids and/or amino acid regions outlined as conserved in Figure 5 are present in β-expansins of the invention as determined by the Clustal alignment algorithm.

Amino acids other than those indicated as conserved will differ among the β-expansins of the invention so that the percent protein similarity between any two β-expansins may vary and may be, for example, 28.5% or up to 60% as determined by using alignment by the Cluster Method and basing similarity on the MEGALIGN algorithm. For purposes of the present invention, any protein that has the conserved regions defined in Figure 5 and is capable of inducing cell wall extension and stress relaxation as described herein is within the scope of the present invention, even if such a protein is not naturally found and is made according to methods of recombinant technology, provided that such a protein is not an α-expansin. With respect to the secondary structure, β-expansins of the invention may have a secondary structure as shown in Figure 1B.

β-expansins may be isolated from both monocotyledon and dicotyledon plants. However, β-expansins produced using recombinant DNA technology are also within the scope of the present invention. In one embodiment, β-expansin is of a dicotyledon origin, i.e., it has an amino acid sequence as originally found in a dicotyledon plant, preferably other than soybean.

In another embodiment, β-expansins of the invention are class I pollen allergens such as, for example, Zea mI and others listed in Figure 4.

In one preferred embodiment, β-expansin is a vegetative homolog of a class I grass pollen allergen. More preferably, a vegetative homolog is not of soybean origin, i.e., it does not have an amino acid sequence of the soybean vegetative homolog. Most preferably, the vegetative homolog of the invention is of *Arabidopsis* or rice origin.

An example of a vegetative homolog of the invention is an *Arabidopsis* vegetative homolog, which may have the amino acid sequence of SEQ ID NO:9 (corresponding to nucleotide cDNA sequence deposited with Genbank, U95967). Other vegetative homologs of *Arabidopsis* having a different amino acid sequence are also within the scope of the present invention.

Another example of a vegetative homolog is a rice vegetative homolog, which may have the amino acid sequence of SEQ ID NOS:10, 11, 12, 13, 14 and 15. In one embodiment, the rice vegetative homolog is a full length polypeptide, i.e., it is of the same length as originally present in rice tissues.

Function-conservative variants of β-expansins for which amino acid sequences are specifically disclosed herein (e.g. *Arabidopsis* and rice) are also within the scope of the present invention. A "function-conservative variant" of SEQ ID NO:9, for example, is a polypeptide which has at least 60% amino acid identity as determined by BLAST or FASTA algorithms, preferably at least 75%, most preferably at least 85%, and even more preferably at least 90%, and which has the property to function as a β-expansin.

In one embodiment, a β-expansin of the invention is a full length polypeptide. For purposes of the present disclosure, a "full length polypeptide" indicates that the β-expansin has the same number of amino acids as the polypeptide originally found in plant tissues. However, continuous fragments of β-expansins are also within the scope of the invention. A "continuous fragment" is a fragment of a β-expansin polypeptide without an internal deletion. Such fragments are at least 20 amino acids long, preferably at least 100 and most preferably at least 200 amino acids long. The fragments of the invention have the property of altering physical properties of a plant cell wall, which can be determined by stress relaxation and wall extension assays. These assays are well known in the art and are described in the Example. The crucial residues in β-expansins are those between positions 55 and 238 in Figure 5, that is, starting with "TWYG" and ending with the "W" at position 238. The most diagnostic motifs are the conserved GGACG box at position 69, the conserved cysteines (C) at positions 101, 104, 109, and 176, the HFD region of the HFDLSG box at position 140, and the tryptophans (W) at positions 227, 234, and 238.

β-expansin polypeptide fragments that do not have the property to function as β-expansins but contain at least one of the conserved regions shown in Figure 5 (and listed above) are also within the scope of the present invention. Such polypeptide fragments are useful for raising antibodies which can then be used to identify and purify other β-expansins.

"Purification" of a β-expansin polypeptide refers to the isolation of the polypeptide in a form that allows its activity to be measured without interference by other components of the cell in which the polypeptide is expressed. Methods for polypeptide purification are well-known in the art, including, without limitation, preparative disc-gel electrophoresis, isoelectric focusing, HPLC, reversed-phase HPLC, gel filtration, ion exchange and partition chromatography, and countercurrent distribution. For some purposes, it is preferable to produce the polypeptide in a recombinant system in which the protein contains an additional sequence tag that facilitates purification, such as, but not limited to, a polyhistidine sequence. The polypeptide can then be purified from a crude lysate of the host cell by chromatography on an appropriate solid-phase matrix. Alternatively, antibodies produced against the β-expansin protein or against peptides derived therefrom can be used as purification reagents. Other purification methods are possible.

The present invention also relates to a composition containing a β-expansin polypeptide. The composition has the property of altering the physical characteristics of a plant cell wall or of any material containing such cell walls (e.g. paper, textile). Preferably, the composition contains an acid medium. Preferably, the pH of the acid medium is in the range of 3.0-5.5 and additionally may comprise a sulfhydryl reducing agent. The pH range is more preferably about 3.5-5 and most preferably is about 4.0. Suitable acid buffers include acetate, citrate, and other organic acids.

Buffer concentrations in the composition of the invention are preferably from about 20 to about 100 mM. In other embodiments of the invention at least 1 mM or at most 500 mM is used. Urea, for example at about 1-2 M, may act synergistically with expansins. Calcium chelators, such as EGTA, EDTA, CDTA, at for example about 1-50 mM can aid expansin action. Thiol reductants such as dithiothreitol or bisulfite, for example at about 1-10 mM may also be used. However, the only essential ingredient is the expansin protein (for example at a concentration of about 1-10 micrograms per mL). In one embodiment, at least 0.1 micrograms per mL may be used (higher than 10 micrograms per mL is very effective, but may be wasteful of the protein).

The present invention also relates to polynucleotides encoding the polypeptides of the invention. A "polynucleotide" is intended to include double or single stranded genomic and cDNA, RNA, any synthetic and genetically manipulated polynucleotide, and both sense and anti-sense polynucleotide (although only sense stands are being represented herein). This includes single- and double-stranded molecules, i.e., DNA-DNA, DNA-RNA and RNA-RNA

hybrids, as well as "protein nucleic acids" (PNA) formed by conjugating bases to an amino acid backbone. This also includes nucleic acids containing modified bases, for example thio-uracil, thio-guanine and fluoro-uracil.

Polynucleotides encoding β-expansins of the invention may be isolated from both monocotyledon and dicotyledon plants. Other plant sources, such as gymnosperms, ferns and mosses, are also within the scope of the present invention. However, β-expansin-encoding polynucleotides produced by using recombinant DNA technology are also within the scope of the present invention. In one embodiment, a polynucleotide encodes a β-expansin of a dicotyledon origin, i.e., it has a nucleotide sequence identical to the one originally found in a dicotyledon plant, preferably other than soybean.

In another embodiment, polynucleotides of the invention encode class I pollen allergens such as, for example, Zea MI and others listed in Figure 4.

In one preferred embodiment, a polynucleotide of the invention encodes a vegetative homolog of a class I grass pollen allergen. In one embodiment, a polynucleotide encodes a vegetative homolog not of soybean origin, i.e., it does not encode an amino acid of the soybean vegetative homolog DNA, cDNA or RNA. Preferably, polynucleotides of the invention encode vegetative homologs of *Arabidopsis* or rice origin.

An example of a polynucleotide encoding an *Arabidopsis* vegetative homolog is presented herein as SEQ ID NO:1 (deposited with Genbank, U95967). Polynucleotides encoding other vegetative homologs of *Arabidopsis* and having a nucleotide sequence different from SEQ ID NO:1 are also within the scope of the present invention.

Another example of a protein encoding a vegetative homolog, is a polynucleotide encoding a rice vegetative homolog, which may have the nucleotide sequence of SEQ ID NOS:2 (Genbank, U85968), 3, 4, 5, 6, and 7. In one embodiment, the polynucleotide encodes a full-length rice vegetative homolog, i.e., of the same length as originally present in rice tissues.

Sequence-conservative and function-conservative variants of polynucleotides encoding β-expansins are also within the scope of the present invention. "Sequence conservative variants" are those in which a change of one or more nucleotides in a given codon position results in no alteration in the amino acid encoded at that position. "Function-conservative variants" are those in which a given amino acid residue in a β-expansin has been changed without altering the overall conformation and function of the polypeptide, including, but not limited to, replacement of an amino acid with one having similar physico-chemical properties (such as, for example, acidic, basic, hydrophobic, and the like). Amino acids which

have similar physico-chemical properties are well known in the art. For example, arginine, histidine and lysine are hydrophilic-basic amino acids and may be interchangeable. Similarly, isoleucine, a hydrophobic amino acid, may be replaced with leucine, methionine or valine. Function conservative variants of β-expansins may encode at least some of the conserved amino acids/regions represented in the alignment in Figure 5. The crucial amino acid residues are pointed out above.

In one embodiment. β-expansin polynucleotide of the invention is a full length polynucleotide. For purposes of the present disclosure, a "full length polypeptide" indicates that the β-expansin has the same number of nucleotides as the polynucleotide originally found in plant tissues. However, continuous fragments of β-expansin polynucleotides are also within the scope of the invention. A "continuous fragment" is a fragment of a β-expansin polynucleotide without an internal deletion. Such fragments are at least 60 nucleotides long, preferably at least 300 nucleotides and most preferably at least 600 nucleotides long. The polynucleotide fragments encode polypeptides that have the property of altering physical properties of a plant cell wall, which can be determined by stress relaxation and wall extension assays.

The polynucleotides of the invention may be isolated directly from cells using appropriate labelled probes containing, for example, regions of high conservation among β-expansins.

Alternatively, PCR can be used to produce the polynucleotides of the invention, using either chemically synthesized strands or genomic material as templates. Primers used for PCR can be synthesized using the sequence information provided herein and can further be designed to introduce appropriate new restriction sites, if desirable, to facilitate incorporation into a given vector for recombinant expression.

The polynucleotides of the present invention may be flanked by natural regulatory sequences, or may be associated with heterologous sequences, including promoters, enhancers, response elements, signal sequences, polyadenylation sequences, introns, 5'- and 3'- noncoding regions, and the like. The nucleic acids may also be modified by many means known in the art. Non-limiting examples of such modifications include methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, and internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoroamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.).

Polynucleotides may contain one or more additional covalently linked moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), intercalators (e.g., acridine, psoralen, etc.), chelators (e.g., metals, radioactive metals, iron, oxidative metals, etc.), and alkylators. The polynucleotides may be derivatized by formation of a methyl or ethyl phosphotriester or an alkyl phosphoramidate linkage. Furthermore, the polynucleotide sequences of the present invention may also be modified with a label capable of providing a detectable signal, either directly or indirectly. Exemplary labels include radioisotopes, fluorescent molecules, biotin, and the like.

The invention also provides nucleic acid vectors comprising the polynucleotides of the invention or derivatives or fragments thereof. A large number of vectors, including plasmid and fungal vectors, have been described for replication and/or expression in a variety of eukaryotic and prokaryotic hosts. Non-limiting examples include pKK plasmids (Clontech), pUC plasmids, pET plasmids (Novagen, Inc., Madison, WI), pRSET or pREP (Invitrogen, San Diego, CA), T-DNA in *Agrobacterium*, and many appropriate host cells, using methods disclosed or cited herein or otherwise known to those skilled in the relevant art. Recombinant cloning vectors will often include one or more replication systems for cloning or expression, one or more markers for selection in the host, e.g. antibiotic resistance, and one or more expression cassettes. Suitable host cells may be transformed/transfected/infected as appropriate by any suitable method including electroporation, $CaCl_2$ mediated DNA uptake, fungal infection, microinjection, microprojectile-mediated transformation, Agrobacterium-mediated transformation, or other established methods.

Appropriate host cells include bacteria, archaebacteria, fungi, especially yeast, and plant and animal cells. Of particular interest are *E. coli, B. subtilis, Saccharvmyces cerevisiae, Sacchaionyces carlsbergensis, Schizosaccharomyces pombi*, SF9 cells, C129 cells, 293 cells, *Drosophila* cell lines, *Neurospora, Pichia*, and CHO cells, COS cells, HeLa cells, and immortalized mammalian myeloid and lymphoid cell lines. Preferred replication systems include M13, ColEl, SV40, baculovirus, lambda, adenovirus, and the like. A large number of transcription initiation and termination regulatory regions have been isolated and shown to be effective in the transcription and translation of heterologous proteins in the various hosts. Examples of these regions, methods of isolation, manner of manipulation, etc. are known in the art. Under appropriate expression conditions, host cells can be used as a source of recombinantly produced β-expansins or derived peptides and polypeptides. For

plant transformation, DNA may be cloned into casettes based on T-DNA plasmids, propagated in *E. coli* or *Agrobacterium*, and used to stably transform plants by the Agrobacterium method. Alternatively, DNA may be inserted into suitably modified plant viruses, such as tobacco mosaic virus, and used to produce recombinant protein by infection of tobacco plants or other sensitive plant species.

Vectors may also include a transcription regulatory element (a promoter) operably linked to the β-expansin sequence. The promoter may optionally contain operator portions and/or ribosome binding sites. Non-limiting examples of bacterial promoters compatible with *E. coli* include: *trc* promoter, alpha-lactamase (penicillinase) promoter; lactose promoter; tryptophan (trp) promoter; arabinose BAD operon promoter; lambda-derived P1 promoter and N gene ribosome binding site; and the hybrid tac promoter derived from sequences of the trp and lac UV5 promoters. Non-limiting examples of plant promoters include: CaMV 35S, PR1, PR, auxin-inducible promoter, ethylene-inducible promoter, heat-shock promoter, seed storage protein promoter.

Non-limiting examples of yeast promoters include: 3-phosphoglycerate kinase promoter, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter, galactokinase (GALI) promoter, galactoepimerase promoter, and alcohol dehydrogenase (ADH) promoter. Suitable promoters for mammalian cells include without limitation viral promoters such as that from Simian Virus 40 (SV40), Rous sarcoma virus (RSV), adenovirus (ADV), and bovine papilloma virus (BPV). Mammalian cells may also require terminator sequences and poly A addition sequences, and enhancer sequences which increase expression may also be included. Sequences which cause amplification of the gene may also be desirable. Furthermore, sequences that facilitate secretion of the recombinant product from cells, including, but not limited to, bacteria, yeast, and animal cells, such as secretory signal sequences and/or prohormone pro region sequences, may also be included.

The present invention further relates to a method of altering the physical properties of a plant cell wall or any product containing plant cell walls, such as for example paper or textile. Given its properties to affect the plant cell wall, β-expansins of the invention find use in a number of industries. For example, β-expansins can be used in the paper industry for paper recycling.

The paper products industry employs 750,000 workers and is a $60-billion industry in the U.S. alone (plus $40 billion in retail sales). Recycling is a growing concern and will prove more important as the nation's landfill sites become more scarce and more expensive.

The advantages of using expansins for paper recycling include the following: the protein is nontoxic and environmentally innocuous; it could substitute for current harsh chemical treatments which are environmentally noxious. The protein is effective on paper products which are now recalcitrant to current recycling processes. Its use could expand the range of recyclable papers. Because the protein acts at moderate temperature and in mild chemical environments, degradation of paper fibers during recycling should be reduced. This should allow for recycled paper fibers with stronger mechanical properties and with the ability to be recycled more often than is currently practical. Moreover, savings in energy costs associated with heating and beating the paper may be realized.

Other modes of application of β-expansins include production of virgin paper. Pulp for virgin paper is made by disrupting the bonding between plant fibers. Beta-expansins may be useful in the production of paper pulp from plant tissues. Use of expansins could substitute for harsher chemicals now in use and thereby reduce the financial and environmental costs associated with disposing of these harsh chemicals. The use of expansins could also result in higher quality plant fibers because they would be less degraded than fibers currently obtained by harsher treatments.

Beta-expansins may be also used to make harsh plant fibers, such as wood fibers, accessible as a biomass source for alcohol production. To achieve this result, β-expansins may be added alone, or in combination with α-expansins, to an alcohol manufacturing process. Alternatively, a plant intended as a source for making alcohol may be transformed with the polynucleotides of the invention hence making a plant having softer fibers that are easier to process. Methods for introducing polynucleotides of the invention into plant cell, and regenerating plants therefrom are well known in the art and are described, for example, in: Plant Molecular Biology, Ed. R.R.D. Croy, Bios Scientific Publishers, Academic Press, 1993. Beta-expansins of the invention may also be used to alter the growth behavior of plants transformed with a β-expansin encoding polynucleotide.

In another embodiment, the invention relates to a method for identifying, isolating and purifying the β and α-expansins of the invention. The method is based on the findings described herein that βp and α-expansins contain conserved sequences as shown in Figures 5 and 6. New expansins may be identified by assaying crude extracts of plant, fungal, or other origin for their ability to induce extension (creep) of cell walls from plants. Suitable plant walls materials include, but are not limited to, frozen/thawed/heat-inactivated specimens from cucumber hypocotyls or grass coleoptiles clamped under tension in an extensometer and

incubated in an acidic buffer, such as 50 mM sodium acetate, pH 4.5. Active extracts may be further purified by combining extensometer assays with protein fractionation techniques such as HPLC, electrophoresis, and selective precipitation with ammonium sulfate, polyethylene glycol, antibodies, and other affinity matrices. In this way, new proteins with expansin activity may be identified and purified. New expansin genes may be cloned in many standard ways, such as the use of polymerase chain reaction (PCR) to amplify gene fragments or cDNA fragments, using primers based on the conserved amino acid residues shown in Figures 5 and 6. Alternatively. cDNA and genomic libraries made from plants, fungi or other biological materials may be synthesized and screened at low stringency (e.g. hybridization and washing in 3X SSC at 50°C using a nucleotide sequence encoding the conserved parts of the expansin protein).

The invention is further described in the following non-limiting example.

EXAMPLES

EXAMPLE I

Protein Structure Analysis.

Dot plots were calculated with Antheprot (McQueen-Mason, S. et al., (1988) CABIOS 5:159-160), using the unity matrix, a window size of 15 and a similarity threshold of 10. Secondary structure predictions were made with the pro-rum PHD via its mail server (Rost, B. (1996) *Meth. Eirzvrnol.* 226:524-539). Hydrophobic cluster analysis used the program PCHCA (B. Boutherin, S. Lavaitte, B. Henrissat; Centre de Recherches sur les Macromolécules Végétales, CNRS, Grenoble, France) to make the initial two dimensional map and standard techniques to identify clusters (Henrissat. B., et al., (1995) *Proc. Natl. Acad. Sci. USA* 92:7090-7094; Lemesle-Varloot, L., et al., (1990) *Biochimie* 72:555-574).

Protein Extraction, Purification and Analysis.

Pollen from greenhouse-grown *Zea mays L.* plants was collected daily, sifted to remove debris, and frozen at -80 C. Twenty g of pollen was thawed, extracted at 4 C for 1 h in 80 mL 0.125 M $NaC0_3$, centrifuged to remove pollen debris, and the supernatant was dialyzed against 10 mM sodium acetate, pH 5.5 or 4.5. Prior to rheology assays, the extract was typically diluted with 4 volumes of 50 mM sodium acetate and adjusted to pH 4.5.

For purification, pollen extract was prepared as above without the dialysis step and desalted on a Bio-Gel P-10 column pre-equilibrated with 10 mM MES, pH 6.0. The

desalted fraction was brought to 60 mM NaCl, and 5 mL (typically 5-10 mg protein per mL) was loaded onto a 2-mL CM-Sepharose column pre-equilibrated with 60 mM NaCL.10 mM MES, pH 6.0. Protein was eluted with a pH gradient and salt steps (0-10 min: isocratic in 60 mM NaCl, 10 mM MES, pH 6.0; 10-75 min: continuous gradient to 60 mM NaCl, 10 mM HEPES. pH 8.5:75-100 min: NaC1 increased in steps to 70 mM, 90 mM, 110 mM, and 220 mM (in 10 mM HEPES, pH 8.5). Fractions were desalted on a 10-kD or 30-kD Centricon microconcentrator prior to further testing.

Proteins were quantified calorimetrically with Coomassie Protein Assay Reagent (Pierce, Rockford, IL) and analyzed by 15% SDS-PAGE and western blots using standard procedures (Li, Z.-C., et al. (1993) *Planta* 191:349-356). Gels were electroblotted onto nitrocellulose membrane and blocked with 10% horse serum in phosphate-buffered saline containing 0.05% Tween-20. To detect expansins, rabbit polyclonal antibody raised against purified cucumber "S1" expansin protein (Li 1993) was used at 1,000:1 dilution and subsequently detected using goat anti-rabbit IgG-conjugated alkaline phosphatase. Mouse monoclonal antibody against Lol pI(4) was used at 5,000:1 dilution to detect group I allergens.

Rheology Assays.

Maize silks were obtained from greenhouse-grown plants: coleoptiles of wheat (*Triticum aestiviun L.*, cv. Pennbar) and hypocotyls of cucumber (*Cucumis sativus L.*, cv. Burpee Pickler) were obtained from 4- to 5-day-old etiolated seedlings germinated in moist vermiculite (Cosgrove, D. J. (1989) *Planta* 177:121-130). For creep reconstitution experiments, 1-cm segments were cut from the apical growing region, frozen at - 20 C, thawed, abraded with carborundum slurry, heat inactivated and clamped in constant-load extensometers, as described previously (Cosgrove 1989). To compensate for the varying thickness of the wall specimens, 5-g weights were used to keep the silk walls under constant tension. whereas 20-g weights were used for the coleoptile and hypocotyl walls. For the stress relaxation measurements, the walls were pretreated for 10 min in either buffer or maize pollen extract, then stored on ice prior to extension and stress-relaxation measurements (Cosgrove 1989). Maximal force equivalents for the stress relaxation assays were 5 for silks, 20 g for coleoptile and hypocotyls.

When GenBank and SwissProt databases were searched using the BLAST and FASTA programs (Altschul, S. F., et al., (1990) *J. Mol. Biol.* 215:403-410; Pearson. W. R. et al., (1988) *Proc. Natl Acud. Sci. USA* 85:2444-2448), the only protein sequences with significant similarity to expansins were the group I pollen allergens and their homologs. Dot plots and sequence alignments show that expansions (hereafter called α-expansins) and group I allergens have short regions of conservation distributed throughout most of the protein backbone (Fig. IA); these consist, notably, of five stretches of 15 amino acids with 40 to 53% identity (identified with the nos. 1 to 5 in Fig. IA). The domains conserved between the specific combination of α-expansin Cs-EXP1 and the pollen allergen Lol pI (Fig. IA) are also highly conserved within both groups of proteins. Likewise. both groups of proteins have hydrophobic signal peptides at the amino termini, characteristic of secreted proteins. Overall, the proteins share only 20-25% amino acid identity.

Despite this low sequence similarity, about 75% of the two proteins are predicted to have the same secondary structure, consisting mostly of loop regions (-60%), with a small proportion of α strand (-25%) and α helix (- 15%) (Fig. 1B). These structural predictions were made with the PHDsec program (Rost, B. et al., (1994) *Proteins* 19:55-77; Rost, B. (1996) *Meth. Enzymol.* 226:524-539), using eight aligned group I allergens to predict the allergen structure and 11 aligned α-expansin homologs to predict the structure of α-expansion. While the accuracy of this prediction method is said to be better than that of other methods (Rost 1994), the important point to be made here is not that the predictions closely approximate the true structure of the proteins, but rather that the two predicted patterns closely resemble each other, despite the low sequence similarity. Likewise, the structural similarities between α-expansins and group I allergens is supported by hydrophobic cluster analysis (Fig. 1C). This method uses a two-dimensional display of amino acids to identify spatial patterns of hydrophobic residues and other motifs that correspond to secondary structure elements and is useful for recognizing related proteins with low sequence similarity (Henrissat 1995; Lemesle-Varloot 1990). Hydrophobic cluster analysis indicates that α-expansins and group I allergens are structurally congruent throughout most of their protein backbones. Six conserved cysteines can be identified, suggestive of a common pattern of disulfide bond formation and protein folding. An additional cysteine pair that is strictly conserved in the α-expansins [cys-105 and cys-119 in Cs-EXPl] is missing in the pollen allergens. From the foregoing observations and

similarities, Applicant hypothesized that group I allergens might have α-expansin-like biochemical activities.

Notwithstanding these structural similarities, α-expansins and group I allergens have notable differences in certain properties, suggestive of divergent biological functions. α-Expansin proteins are found in low abundance even in rapidly growing tissues where they are specifically expressed; they are not readily soluble in solutions of low ionic strength, are not glycosylated, and are tightly bound to cell walls (McQueen-Mason 1992; McQueen-Mason, S. et al., (1995) *Plant Physiol.* 107:87-100; Cosgrove, D. J. (1996) *BioEssays* 18:533-540.). In contrast, group I allergens are found in high abundance in pollen, are highly soluble in dilute solutions, are glycosylated, and apparently do not bind tightly to the pollen wall (Marsh, D. G. (1975) in *Allergens and the genetics of allergy*, ed. Sela. M. (Academic Press, New York), pp. 271-291; Knox, R. B., et al., (1993) in *Pollen allergens: botanical aspects*, eds. Kraft, D. L, Sehon, A. (CRC Press, Boca Raton), pp. 31-38). These differences suggest that the function of the group I allergens may be to loosen or expand the cell walls of the stigma and style to allow penetration of the pollen tube through these tissues. The grass pollen tube grows by tip growth to force its way between the tightly pressed cell walls of the stigma before entering the stylar track, where growth of the pollen tube involves further intrusive growth through and between cell walls (Heslop-Harrison, Y., et al., (1984) *Acta Bot. Neerl.* 33:81-99). Secretion of cell-wall loosening or expanding agents with expansin-like properties would presumably aid invasion of the pollen tube into the maternal tissues.

EXAMPLE 2

To test whether group I pollen allergens may loosen or expand cell walls, Applicant extracted protein from maize (*Zea mays*) pollen, which contains the group I allergen Zea mI (Broadwater, A. H., et al., (1993) *Gene* 131:227-230; Bedinuer, P. A., et al., (1994) in *Molecular Studies of pollen development in maize*, eds. Stephenson. A. G. & Kao, T.-h., (American Society of Plant Physiologists, Rockville, MD.), pp 1-14), and assayed its effects on the wall rheology of maize silks, which are the receptive stigmas and styles of the maize flower. Maize was used for these experiments because it is easy to collect large quantities of maize pollen and because the large size of the maize silk facilitates rheological assays. For these assays, silk walls were prepared so as to inactivate endogenous proteins and they were then clamped either at constant force to measure extension behavior or at

constant extension to measure stress relaxation behavior (McQueen-Mason 1994; Li 1993). Addition of the maize pollen extract induced rapid, irreversible extension (creep) of the silk walls when tested in constant-force extensometers (Fig. 2A). Likewise, the pollen extract enhanced stress relaxation of the silk walls over a large range of times (Fig. 2B). Both of these rheological effects are unique characteristics of expansion action (McQueen-Mason 1992; McQueen-Mason 1995; Cosgrove 1996). Moreover, these rheological effects required an acidic pH (<5.5), likewise similar to the action of expansins. These results demonstrate that maize pollen can release a potent expansin-like activity. They also give direct support to suggestions that proteins secreted by pollen may alter the walls of receptive tissues (Wing, R. A. et al., (1990) *Plant Mol. Biol.* 14:17-28; Turcich, M.P., et al., (1993) *Plant Mol. Biol* 23:1061-1065; Mascarenhas, J. P., (1990) *Am. Rev. Plant Physiol. Plant Mol. Biol.* 41:317-338).

Despite its expansin-like activity and the limited amino acid similarity between the allergens and α-expansins, the pollen extract did not contain proteins recognized by anti-expansin antibodies (Fig. 3B). These antibodies recognize α-expansins of both dicot and monocots (Li 1993; Keller, E., et al., (1995) *Plant J.* 8:795-802; Wu. Y., et al., (1996) *Plant Physiol.* 111:765-772). Other properties also belie the possibility of a cryptic presence of an α-expansin in the pollen extract: The pollen activity was readily soluble in solutions of low ionic strength, whereas higher salt concentrations are needed to extract and maintain solubility of α-expansins. Concentrations of NaCl greater than 200 mM strongly inhibited the creep activity of the pollen extract, whereas at least two-fold higher concentrations were required to inhibit α-expansin activity. Microcrystalline cellulose (Avicel, 10 mg/mL) depletes α-expansin solutions of creep activity by binding α-expansins and removing them from solution (McQueen-Mason 1992; McQueen-Mason 1995), but this was not possible with the pollen activity. Applicant concludes therefore that the maize pollen extract does not contain a classical α-expansin protein.

Consistent with previous work (Broadwater 1993), the pollen extract did contain Zea mI, a group I allergen recognized by antibodies raised against the rye grass pollen allergen Lol pI (Fig. 3C). The pollen extract was fractionated on a carboxymethyl Sepharose column, and fractions were assayed by immunoblot, SDS-PAGE, and wall extension assays (Fig. 3D-G). Fractions testing positive for group I allergens by immunoblotting possessed significant wall extension activity, whereas fractions testing negative in the immunoblot assay lacked expansin-like wall extension activity. A fraction

highly purified for Zea ml (Fig. 3E,F) tested positive in the wall extension assay (Fig. 3G). Applicant therefore concludes that Zea ml possesses expansin-like wall loosening or expanding activity.

Late-eluting fractions (i.e. at 80-95 min in Fig. 3D) also contained isoforms of Zea mI and exhibited potent creep activity (not shown), but they also contained additional proteins. Some pollen fractions caused sudden wall breakage (unlike expansins) or acted synergistically when added to pure Zea ml fractions (data not shown); these fractions may contain pectate lyases or other wall degradative enzymes (Turcich 1993: Mascarenhas 1990).

Further work showed that the maize pollen extract was more effective as a wall loosening or expanding agent with grass cell walls than with dicot cell walls. For example, the pollen extract had a marked effect on the creep (extension) and stress relaxation of coleoptile walls from young grass seedlings (Fig. 2C, D), but its rheological effects on hypocotyl walls from cucumber seedlings were small. At the same concentration that proved very effective on grass walls (i.e. at 1:4 dilution), the pollen extract had a barely detectable effect on wall creep and stress relaxation of cucumber walls (data not shown). Even at 5-fold higher concentration (i.e., undiluted pollen extract), the activity seen using cucumber walls was only about one quarter the activity found using the 20% extract on grass walls (Fig. 2E. F).

This selectivity for grass walls complements the action of α-expansins, which appear to induce creep more effectively in dicot walls than in grass coleoptile walls (McQueenMason 1992). Even though (α-expansins are found in grass coleoptiles (Shcherban 1995), they proved more effective on dicot walls than on grass coleoptile walls, at least as assayed by reconstitution assays of wall creep (14).

Similar, though less extreme, results were found in creep reconstitution assays with wall specimens from maize roots (W u 1996) and rice internodes (Cho, H.-T., et al.. (1997) *Plant Physiol.* in press). In this context. it is notable that grass walls are unusual in composition, being relatively poor in pectins and xyloglucans and rich in glucuronoarabinoxylans and (1 →3), (1→4)-α-D-glucans, when compared with walls of other angiosperms, including other monocots (Carpita. N. C., et al. (1993) *Plant Journal* 3:1-30). It seems likely that α-expansins and Zea mI act on different components of the wall, which may differ in abundance and in their role in wall mechanics in dicots versus grasses.

Additional insight into the functional significance of the Group I allergens and their homologs may be gained from analysis of the protein and DNA databases. Group I allergens have been identified in the pollen of many grass species (Smith. P. M., et al., (1994) *Mol. Immunol.* 31:491-498), but not in pollen of species outside the grass family, including ragweed and other species that elicit potent pollen allergies. Neither have they been identified in monocots outside the grass family. Applicant tested pollen extracts from petunia (a dicot) and lily (a monocot, but not a grass) for wall extension activity, with negative results. These observations suggest that grasses may be unique in expressing high levels of these wall-loosening or expanding proteins in pollen.

EXAMPLE 3

An analysis of the rice and *Arabidopsis* cDNA databases shows that expression of this gene family is not limited to pollen. The rice EST (Expressed Sequence Tag) collection currently contains 18 partially sequenced cDNA entries that are close homologs to the group I pollen allergens (e.g., long stretches with 60% identity and 80% similarity at the amino acid between Lol pI and the rice EST homologs). The 18 cDNAs fall into 7 distinct sequence classes, represented by Genbank accession numbers D41180, D24261, D46769, D39144. D24972, D40180, D48180. As they are, all expressed in young seedlings without flowers, these cDNAs cannot be from pollen, and so they are referred to as vegetative homologs of the group I allergens. The *Arabidopsis* EST collection currently contains at least 1 homolog of the pollen allergens (Genbank accession number Z37641), which is likewise expressed in young seedlings without flowers. Additionally, ciml, a cytokinin-induced gene expressed in soybean cell cultures (Crowell, D. N. (1994) *Plant Mol. Biol.* 25:829-835), is also a vegetative homolog of the group I allergens. Vegetative homolog of the group I allergens from the rice and Arabidopsis EST collections were sequenced. These sequences were used together with related sequences in Genbank, to construct a phylogenetic tree for α-expansins and group I allergens (Fig. 4). The tree shows two deeply branched families, with the vegetative homologs of the group I allergens occupying a position intermediate between the group I allergens and α-expansins. Because α-expansins and group I allergens have wall-loosening or expanding activity, the vegetative homologs of the group I allergens are also expected to possess expansin-like properties.

To test this idea, an attempt was made to identify the vegetative homologs of group I allergens by western blots of wall proteins extracted from grass seedlings using monoclonal

antibody directed against Lol pl, but without success (not shown). This is consistent with previous results (Bedinuer 199=1) and likely indicates that the major antigenic determinants of the group I pollen allergens are not conserved in their vegetative homologs. Our attempts to express recombinant expansins and group I allergens in *E. coli* have so far failed to result in active protein, evidently because of faulty disulfide bond formation (unpublished results of M. Shleh and D.J. Cosgrove). Thus, it remains to be seen how the activity of the vegetative homologs of the allergens compares with the pollen allergens and with α-expansins.

The experimental results reported here, as well as the database observations, suggest that the group I grass pollen allergens and their homologs in vegetative tissues constitute a second multi-gene family of expansins that function as wall loosening or expanding agents in plants, preferably in angiosperms. It is proposed that this family be referred to as beta-expansins, and the original family of expansins henceforth be referred to as α-expansins. The two families of expansins exert similar biophysical effects on the wall (i.e., they induce prolonged creep and stress relaxation in a pH-dependent manner), but apparently they interact with different components of the wall. The limited sequence similarity between these two families of expansins gives obvious targets for future studies of active sites and functional domains in these proteins.

In the grasses, the group I pollen allergens represent a subset of the beta-expansin family that appears to have assumed a specialized role during pollination, most likely for wall loosening or expanding of the maternal tissues for rapid pollen tube penetration. This idea is directly supported by our results, which show that these proteins have potent rheological effects on the walls of the grass stigma and style, where they are naturally released in abundance by the grass pollen. An additional possibility is that group I allergens are involved in pollen tube, where surface expansion occurs.

Determination of the in-vivo functions of beta-expansins in vegetative tissues will require further work. A potential wall-loosening or expanding role for the beta-expansin ciml is consistent with induction of its expression by cytokinin (Crowell 1994), which stimulates cell proliferation and growth in soybean cell cultures. The large number of distinct beta-expansins expressed in rice seedlings suggest that beta-expansins have assumed multiple roles in grass seedling development, perhaps as agents

controlling different types of cell growth, wall dissolution and separation, or other processes where wall pliancy is important.

EXAMPLE 4

The beta-expansin gene may be isolated from any plant genomic DNA by (i) obtaining a beta-expansin gene fragment using the polymerase chain reaction (PCR) with a series of degenerative primers directed against the 5' and 3' regions of the beta-expansin sequence; and (ii) screening the plant genomic library with the obtained gene fragment as a primer to identify and isolate the full length gene. Degenerative primers can be based on conserved sequences represented in Figure 5, such as for example regions TWYG, GGACG, HFDLSG, and HFD. This experimental approach can also be used with a plant cDNA library.

The genomic DNA (10 ng) may be amplified in a 100 μl reaction containing for example: 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 500 mM KC1. 2.5 U Taq polymerase (Beohringer-Mannheim), 0.2 mM dNTPs and oligonucleotide primers (1 μM each) specific for beta-expansin sequences. PCR may be performed in an Omnigene thermocycler or any other available thermocycler. Amplification condition are generally known and can be optimized using routine experimentation. Other protocols for amplification may be used and are well known to persons of skill in the art. For example, protocols described in U.S. Patents 4,683,195, 4,683,202 and 4,800,159, and in Innis et al., *PCR Protocols*, Academic Press, Inc., San Diego CA, 1990 (each of which is incorporated herein by reference in its entirety) may be used.

The amplification products containing beta-expansin polynucleotides may be gel-purified and ligated into any vector, for example into a pGEM vector. *E. coli* may be transformed with derived recombinant plasmids and cultured overnight at room temperature. Plasmid DNA may be isolated to prepare a probe for screening a cDNA or genomic library using standard methods described in, for example, Sambrook et al., Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, 1989, incorporated herein by reference.

The isolated DNA clone may then be used as a hybridization probe to recover the entire beta-expansin gene from a cosmid or lambda genomic library. The libraries may be prepared according to the methods well known in the art and described in Sambrook et al. The above-described procedure can be followed to isolate beta-expansin genes from any

plant species. Furthermore, based on the conserved regions represented in Figure 6 (for example regions GGACG and HFD), degenerative nucleotides may be constructed and the above procedure may be used to identify and isolate genes encoding alpha-expansins.

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